In vitro study of the antifungal efficacy of zinc oxide nanoparticles against *Fusarium oxysporum* and *Penicillium expansum*

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The antifungal efficiency of Zinc oxide nanoparticles (ZnO NPs) was investigated against two pathogenic fungal species, *F. oxysporum* and *P. expansum*. The two fungi were identified at molecular level by nuclear ribosomal DNA internal transcribed spacer (ITS) identities. They have been submitted to the GenBank with accession numbers of AB753032 and AB753033 for *F. oxysporum* and *P. expansum*, respectively. The antifungal activity of ZnO NPs was found to be concentration dependent. Hence, maximal inhibition of mycelial growth corresponded to the highest experimental concentration (12 mg L⁻¹), where 77 and 100% growth inhibition was observed for *F. oxysporum* and *P. expansum*, respectively. The effect of ZnO NPs on the mycotoxins fusaric acid and patulin production by *F. oxysporum* and *P. expansum*, respectively, was investigated using HPLC quantification. It was observed that ZnO NPs prevented both mycotoxins synthesis in a concentration dependent manner. Fusaric acid was reduced from 39.0 to 0.20 mg g⁻¹ while patulin production was reduced from 14.2 to 1.10 mg g⁻¹ in control and 12 mg L⁻¹ ZnO NPs treated samples, respectively. The scanning electron microscopy (SEM) revealed obvious deformation in the growing mycelia treated with ZnO NPs in *F. oxysporum* which may be the cause of growth inhibition.

Key words: Zinc Oxide Nanoparticles (ZnO NPs), antifungal efficiency, *Fusarium oxysporum*, *Penicillium expansum*.

INTRODUCTION

Fungal growth and pathogenicity are the main cause of considerable economic loss during postharvest and handling of vegetable crop and fruits. *Fusarium oxysporum* is a cosmopolitan fungus that includes pathogenic and saprophytic members. The pathogenic members are best known for causing *Fusarium* wilt diseases of many economically important crops (Tripathi et al., 2009). Until now, synthetic fungicides are used as primary means to control *Fusarium* wilts. While *Penicillium expansum* causes severe postharvest fruit diseases including grey and blue mold even when the most advanced postharvest technologies are applied (Spadaro et al., 2004), it primarily causes the rot of stored apples and pears (Caban as et al., 2009). Furthermore, *P. expansum* is regarded as the major producer of a mycotoxin, patulin, which is commonly found in rotting apples. U.S. Food and Drug Administration (FDA) limits patulin to 50µg/L in apple juices (Moake et al., 2006).

Today a number of synthetic fungicides have been found to cause adverse effects to humans and the environment.
and for this reason many of them have been banned. It is difficult to control fungal growth because fungi have developed resistance to many conventional fungicides such as benzimidazoles and dicarboximides (Elad et al., 1992). Moreover, these traditional fungicides are becoming ineffective due to the development of new physiological races of the pathogens (Ocamb et al., 2007). To overcome resistance, it is important to explore novel antifungal agents, which may replace current control strategies. In recent years, nanoparticle (NP) mate-rials have received increasing attention due to their unique physical and chemical properties, which differs significantly from their conventional counterparts (Stoimenov et al., 2002).

Recent studies have demonstrated antimicrobial efficacy against bacteria, viruses and eukaryotic microorganisms of various NP materials, including silver (Kim et al., 2008; Kumar et al., 2008), copper (Cioffi et al., 2005), titanium dioxide (Kwak et al., 2001), magnesium, gold (Gu et al., 2003), alginate (Ahmad et al., 2006) and zinc oxide (Liu et al., 2009). Highly ionic nanoparticle metal oxides such as MgO, CaO and ZnO nanoparticles (NPs) are unique in that they can be produced with high surface areas and with unusual crystal structures (Klabunde et al., 1996).

Compared to organic materials, inorganic materials such as ZnO possess superior durability, greater selectivity, heat resistance (Padmavathy and Vijayaraghavan, 2008) and the mineral element is essential to human health ZnO NPs also have good biocompatibility to human cells (Padmavathy and Vijayaraghavan, 2008). The antibacterial and antifungal activity of bulk ZnO powders has been already demonstrated (Yamamoto, 2001; Sawai and Yoshikawa, 2004).

In agriculture, zinc compounds are mainly used as fungicides (Waxman, 1998). The 50% lethal dose (LD50) of oral toxicity for ZnO is relatively high reaching 240 mg/kg for rats (South, 2002). It is believed that smaller sizes of ZnO, correlated with its stronger antimicrobial activity (Yamamoto, 2001).

Preliminary studies show that the antibacterial activity of ZnO NPs might be related to the formation of free radicals on the surface of the NPs, and the damage to the lipids in bacterial cell membrane by free radicals, which consequently lead to the leakage and breakdown of bacterial cell membrane (Brayner et al., 2010; Reddy et al., 2007). However, to the best of our knowledge, the effect and mode of action of ZnO NPs on the growth of fungi such as F. oxysporum and P. expansum have not been studied. Therefore, in the current study the anti-fungal activity of ZnO NPs against two important plant pathogenic fungi, F. oxysporum and P. expansum was investigated.

**MATERIALS AND METHODS**

**Nanoparticle materials**

ZnO NP suspensions with size of 70±15 nm were purchased from Sigma Aldrich. An aliquot of ZnO NP suspension was vacuum filtered through an aluminum oxide membrane filter with a 20 nm pore size and 25 mm OD (Anodisc; Whatman Inc., Clifton, NJ, USA), resulting in a NP-free solution. The composition of the NP-free solution was analyzed, and its effect on bacterial growth was examined. The original ZnO NP suspension (12 mg L⁻¹) and NP-free solution were then diluted with potato dextrose agar (PDA, containing per liter the extract from 200 g boiled potato, 20 g glucose and 20 g agar) to make a series of media containing ZnO NPs with concentrations of 0, 2, 4, 6, 8 and 12 mg L⁻¹, respectively and NP-free solution.

**Isolation and identification of fungal isolate**

Fungal samples were taken from different infected plant tissues. The pure culture was maintained on PDA media (Hi media Laboratories Ltd. Bombay, India) at 25±1°C. Identification of the fungal isolates was carried out by morphological and microscopic examination using the identification keys of Moubasher (1993) and Raper and Fennell (1985) followed by nuclear ribosomal DNA internal transcribed spacer (ITS) sequencing. The genomic DNA was isolated by CTAB extraction method using standard protocols (Sambrook et al., 1989). Internal transcribed spacer (ITS) regions were amplified using primers ITS1 and ITS4 with 5'-TCCGTTAGGTGAACCTGCGG and 5'-TCCCTCCTTTGATATGC sequences (White et al., 1990). Amplified products were sequenced using ABI prism DNA sequencer by BigDye terminator method. The sequence obtained has been deposited in the GenBank database. The ITS sequence information was used to match the most closely retrieved fungal isolates with the NCBI BLAST program. The alignments of the ITS sequences were performed using ClustalW, and phylogenetic tree constructed with the maximum parsimony method and MEGA ver. 5 (http://www.megasoftware.net) (Felsenstein, 1985).

**Antifungal test**

F. oxysporum and P. expansum were cultured on PDA at 25°C. Antifungal tests were performed by the agar dilution method (Fraternale et al., 2003) with slight modifications. The autoclaved PDA media with ZnO NPs at concentrations of 0, 2, 4, 6, 8 and 12 mg L⁻¹ and a NP-free solution were poured into the Petri dishes (9 cm diameter). A plug (1 cm) of fungal mycelia was taken from the edge of 7-day-old plate, placed in the center of each Petri dish and incubated at 25°C. The efficacy of ZnO NP treatment was evaluated at the time intervals of 2, 4, 6, 8, 10 and 12 days by measuring the diameter of fungal colonies. All tests were performed in triplicate and the diameters were expressed in centimeters.

**Scanning electron microscopy (SEM)**

SEM was used to examine morphological changes of F. oxysporum and P. expansum hyphae before and after treatment with ZnO NPs. Pieces of mycelial material cut from 7-day-old cultures were inoculated onto the PDA containing 12 mg L⁻¹ ZnO NPs in comparison with control containing no ZnO NPs, followed by incubation for 12 days. Then, pieces of mycelia were cut from the edge of the fungal cultures, and directly subjected to SEM analysis under the environmental mode. SEM images were taken by FEI Quanta 600F Environmental SEM (FEI Company, Hillsboro, OR, USA) at a voltage of 7 or 10 kV and a pressure from 525 to 619 Pa.

**Mycotoxins estimation**

**Fusaric acid Toxin of Fusarium oxysporum**

The production and extraction of fusaric acid was performed accor-
amplification and sequencing of fungal rRNA gene resulted in 620 and 750 bp long nucleotide sequence, which have been submitted in NCBI GenBank (Accession Numbers: AB753032 and AB753033) for *F. oxysporum* and *P. expansum*, respectively. The sequences were compared using BLAST algorithm and the closely related sequences were selected, which confirmed that isolated strains exhibited 98 and 97% similarity with *F. oxysporum* and *P. expansum*, respectively. A phylogenetic tree was generated using the maximum parsimony method and MEGA ver. 5 (Figures 1 and 2).

ZnO NPs are usually present in a form of agglomerates during its manufacturing process (Zhang et al., 2007). Two methods, ultrasonication and addition of dispersants, are often used to break down NP agglomerates. Commonly used dispersants include polyvinylpyrrolidone, polyethylene glycol, and other chemicals (Brayner et al., 2006). Figures 3 and 4 represent the growth of *F. oxysporum* and *P. expansum* cultivated on PDA containing different concentrations of ZnO NPs (0, 2, 4, 6, 8 and 12 mg L⁻¹) and incubated at 25°C for 12 days. Generally, the use of ZnO NPs suspension efficiently inhibited fungal growth of both *F. oxysporum* and *P. expansum*. The average mycelial growth inhibition in *F. oxysporum* has been ranged from 19.3 to 77.5% as ZnO NP concentration increases from 2 to 12 mg L⁻¹ (Figure 3). For *P. expansum*, the reduction rate of fungal growth significantly varied from 25.3 to 100% as the concentration of ZnO NPs increased from 2 to 12 mg L⁻¹ with a near complete inhibition at 6 mg L⁻¹ (Figure 4). The efficacy of ZnO NPs was found to be concentration dependent in both two fungal species. These results suggest that ZnO NPs may disrupt and damage the conidia of fungi. Consequently, the growth was deeply inhibited.

**P. expansum** proved to be more sensitive than *F. oxysporum* to the ZnO NPs treatment. Hence, different antifungal effects may result from different growth morphologies of fungi. **P. expansum** tends to grow more densely on the surface of the medium than *F. oxysporum*, so it has a larger contact area with ZnO NPs than *F. oxysporum***.

**RESULTS AND DISCUSSION**

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Figure 2. Phylogenetic analysis of *Fusarium* spp based on the gene sequences showing the relationship of *F. oxysporum* to other *Fusarium* spp. The phylogenetic tree was constructed based on internal transcribed spacer sequences using the maximum parsimony method on MEGA ver. 5.

Figure 3. Effect of ZnO NPs on the colony diameter of *F. oxysporum*.
Figure 4. Effect of ZnO NPs on the colony diameter of *P. expansum*.

Another possible reason for the difference in antifungal efficacy among fungi could be the constitutive tolerance of each fungus to ZnO NPs. He et al. (2011) found that ZnO NPs at concentrations greater than 3 mmol L\(^{-1}\) can significantly inhibit the growth of *Botrytis cinerea* and *P. expansum*. Sawai and Yoshikawa, (2004) reported the minimum inhibitory concentration of bulk ZnO powder against *Saccharomyces cerevisiae*, *Candida albicans*, *Aspergillus niger*, and *Rhizopus stolonifer* was over 100 mg ml\(^{-1}\) (\(~1.2\) mol L\(^{-1}\)) by an indirect conductimetric assay (Sawai and Yoshikawa, 2004). ZnO NPs show great enhancement in the antimicrobial activity due to their unique properties such as large surface area. However, Kasemets et al. (2009) found nano and bulk ZnO were of comparable toxicity against *S. cerevisiae*.

To investigate the mechanism by which ZnO NPs affect the growth of *F. oxysporum* and *P. expansum*, SEM analysis was employed to examine the structural changes of fungal samples after ZnO NPs treatment. PDA media containing 12 mg L\(^{-1}\) ZnO NPs were prepared, and fungal samples were then inoculated onto the PDA plate and incubated at 25°C for 12 days.

Figure 5 shows the images of mycelia obtained from the edge of *F. oxysporum* and *P. expansum* culture. In the control (untreated sample), hypha with typical net structure and smooth surface were observed. After treatment with 12 mg l\(^{-1}\) ZnO NPs, the hypha lost their smoothness and appeared swollen and crumbled (Figure 5). This indicated that ZnO NPs growth inhibition in *F. oxysporum* and *P. expansum* may be due to deformation in the structure of fungal hypha. SEM has been successfully used to assess morphological changes of microbial cells induced by ZnO NPs (Brayner et al., 2006; Zhang et al., 2007) and fungal hyphae treated with other chemicals (Sharma and Sharma, 2008; Yen et al., 2008). Some studies proposed that ZnO NPs may cause structural changes of microbial cell membrane, causing cytoplasm leakage and eventually the death of bacterial cells (Sawai and Yoshikawa, 2004; Brayner et al., 2006). Endo et al. (1997) have reported that the inhibition of bud growth by Ag-NPs correlates with membrane damage. This report suggests that Ag-NPs inhibit the normal budding process, probably through the destruction of membrane integrity. *F. oxysporum* and *P. expansum* are well-known as mycotoxins producers. Table 1 revealed that the production of mycotoxins by the tested fungi gradually decreased with the increase of ZnO NPs concentrations to reach a minimum value at the highest concentration 12 mg L\(^{-1}\). The total mycotoxins at 12 mg L\(^{-1}\) were recorded to be 0.20 and 1.1 mg g\(^{-1}\) as compared to 39.0 and 14.2 mg g\(^{-1}\) in control, in cases of *F. oxysporum* and *P. expansum*, respectively. This inhibition of mycotoxin synthesis may be attributed to inactivation of certain enzymes in the biosynthesis pathway of these toxins by ZnO NPs.
Figure 5. Scanning electron microscopy images of *F. oxysporum* and *P. expansum* without (A and C) or with (B and D) the treatment of ZnO NPs (12 mg L$^{-1}$).

Table 1. Effect of ZnO nanoparticles at different concentrations on production of mycotoxins (mg g$^{-1}$ dry mass) by *Fusarium oxysporum* and *Penicillium expansum*.

<table>
<thead>
<tr>
<th>ZnO nanoparticle conc. (mg L$^{-1}$)</th>
<th><em>Fusarium oxysporum</em></th>
<th><em>Penicillium expansum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fusaric acid</td>
<td>Patulin</td>
</tr>
<tr>
<td>0</td>
<td>39.00$^a$</td>
<td>14.20$^a$</td>
</tr>
<tr>
<td>2</td>
<td>35.02$^b$</td>
<td>12.32$^b$</td>
</tr>
<tr>
<td>4</td>
<td>13.00$^c$</td>
<td>9.56$^c$</td>
</tr>
<tr>
<td>6</td>
<td>10.51$^d$</td>
<td>4.73$^d$</td>
</tr>
<tr>
<td>8</td>
<td>6.32$^e$</td>
<td>4.15$^e$</td>
</tr>
<tr>
<td>12</td>
<td>0.20$^f$</td>
<td>1.10$^f$</td>
</tr>
</tbody>
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$^a$, significant difference ($P<0.05$) according to Duncan test.

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REFERENCES


